MICROBIOLOGY OF THE SURFACE RIPENING OF BRICK CHEESE[1,2]

The flavor of mild, cured brick cheese is one of its most important characteristics, yet comparatively little is known concerning the causes of the desired flavor or methods of producing it. The characteristic brick flavor has been attributed chiefly to the action of the microbial flora that grows as a "smear" on the surface of the cheese during the early part of ripening. Langhus *et al.* *(12)* reported growth of yeasts, micrococci, and a rod-shaped bacterium in that order, but did not identify them. Iya and Frazier *(9)* identified the film yeasts isolated from brick cheese smears at different factories as species of *Mycoderma.*

The surface flora of other varieties of surface ripened cheeses has been studied, but few of the microorganisms concerned have been identified. Usually, yeasts, micrococci, and bacilli were reported. Grimmer and Aronson *(7)* found the yellow-pigmented *Micrococcus flavus, M. citreus, M. sulfureus,* and *M. aurantiacus* on the surface of Tilsit cheese. Janiak *(10)* found the yeasts on Tilsit and Limburger cheese to be species of *Torula* and *Mycoderma* and one of the rods to be *Bacterium linens. B. linens* has been found in cheese smears by numerous other workers. Recently Hartley and Jezeski *(8)*, working with the slime on curing blue cheese, found yeasts and mold, micrococci, and rods appearing in that order. On cheese ripened at 46-49° F. the rod was *B. erythrogenes,* and on cheese cured at 55-58° F. it was *B. linens.*

The color of the smear on cheese surfaces may vary from yellow through orange to an almost reddish tinge. The yellow color has been attributed, in part at least, to pigmented micrococci, but the orange to reddish brown shades have been attributed to *B. linens* by a long series of workers, including Weigmann *(15)*, Grimmer and Aronson *(7)*, and Albert *et al. (1)*.

A number of workers have concluded that the enzymes of the surface flora of cheese were not significant in the splitting of protein within cheese of the following varieties: brick *(12)*, Gruyère and Tilsit *(11)*, Wilster Marschkäse *(2)*, Belpaese *(16)*, and Trappist *(6)*. Most workers agree, however, that the surface flora contributes to the flavor of the cheese, although the chemical compounds responsible for the flavor have not been identified.

METHODS

Cheeses from which isolations of pure cultures were made came from three factories in Wisconsin, from the Department of Dairy and Food Industries of

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[3] Present address: Canada Malting Co., Ltd., Montreal, Canada.

this University, and from the curing rooms of the Kraft Foods Company of Wisconsin at Beaver Dam. The cheeses were 7 days old when received from outside and were cured to completion in curing rooms here.

Manufacture of cheese. Cheeses to be inoculated on the surface with selected isolated cultures were made in the University Dairy by means of the "sweet curd" method recommended by Buyens *(4)* from milk pasteurized at 162° F. for 16 seconds. Before use, all equipment that would contact the milk or cheese was treated for at least 5 minutes with a hypochlorite solution containing 200 p.p.m. of available chlorine. To increase the number of cheeses that could be cured at once, normal-sized cheeses were halved by means of a sterilized metal follower covered with sterilized Viscon paper, while the cheese was in the hoop and after it had been turned the second time. Because of the increased ratio of surface area to volume, the time of salting was reduced from 24 to 20 hours.

The cheeses rested on sterilized aluminum trays in the curing chamber, which was a low temperature incubator made from a refrigerator. The humidity was kept high by means of sheets of cheesecloth draped along the sides and across the bottom of the refrigerator, with the ends of the sheets resting in water-filled trays. A thin coating of mineral oil prevented an accumulation of ice on the refrigerating unit. The curing temperature was 60° F.

Inoculation of cheese. The inoculation of film yeasts onto the surface of the cheeses was accomplished by the use of pasteurized 22% brine, inoculated with a suspension of the selected yeast and used in lieu of the usual brine or salt bath. When each cheese was removed from the brine it received an additional inoculum of film yeast suspension, of micrococci, of *B. linens,* or of combinations of these organisms. This inoculum was rubbed over the surface of the cheese by an operator wearing sterilized rubber gloves. The cultures for the preparation of inocula were grown originally in broth on a rotary shaker; and the cells were collected from 100 ml. of culture by centrifugation, washed twice with physiological salt solution, and resuspended in 10 ml. of the saline solution.

The yeasts were grown in a broth, hereafter called Iya broth:

KH_2PO_4	0.5 g.	Bacto-Tryptone	5 g.
$NaNH_4HPO_4 \cdot 4H_2O$	1.0 g.	Liver extract*	100 ml.
Cerelose	50.0 g.	Carrot extract*	100 ml.
NaCl	3.0 g.	Tap water	800 ml.

* One pound of ground, fresh beef liver is steamed with 1 l. of distilled water for 30 min. After filtration through several layers of cheesecloth, the extract is bottled and sterilized for 20 min. at 121° C. The carrot extract is prepared in a similar manner.

The micrococci and *B. linens* were grown in a broth of the following formula: 5 g. glucose, 10 g. Bacto-Tryptone, 5 g. Bacto-Yeast Extract, 2 g. K_2HPO_4, 1,000 ml. distilled water; pH 6.9-7.1.

Handling during ripening. Every other day during the 2-week ripening period the cheeses were rubbed and turned, the operator wearing sterilized rubber gloves previously moistened with sterile water.

Sampling during ripening. Samples were taken just before the cheese was handled so that 48 hours of undisturbed growth had been permitted. A thin piece of rind was cut from the upper surface of the cheese with a sterilized scalpel, deposited in a sterilized petri dish, trimmed to an area of 1 sq. cm., and then shaken in 10 ml. of 0.5% sodium citrate solution. After dispersion of the rind further dilutions were made as desired.

To prepare contact slides for the microscopic examination of the surface flora, glass slides were pressed against the surface of the cheese and the adhering material was spread uniformly over the slide with the aid of a loopful of water. The slides were dried, defatted with xylene, fixed with alcohol, and stained by the Hucker modification of the Gram method.

Grading of ripened cheese. After the cheese had been paraffined it was cured at about 40° F. for 6 weeks and then graded by three judges, who evaluated the flavor and total grade of the cheese numerically as follows:

1. Excellent
2. Desirable
3. Satisfactory
4. Objectionable
5. Very objectionable
6. Not salable as original cheese

Grading was within half points, and results were expressed as averages of the points allotted by the respective judges. Special attention was paid to the evaluation of flavor. The authors joined the three judges in this evaluation.

Isolation and identification of cultures. The micrococci and yeasts were isolated by plating in appropriate media and were purified by streaking procedures. No attempt was made to identify the yeasts as to genus. Purified cultures of micrococci were studied by means of methods described in the *Manual of Methods for the Pure Culture Study of Bacteria (14)* and identified as to species on the basis of descriptions in *Bergey's Manual of Determinative Bacteriology (3).* Organisms were tested for their proteolytic ability on milk agar, which was flooded with 1% HCl after incubation. Tests for lipolysis were made on agar containing tributyrin (Bacto-Tryptone, 10 g.; Bacto-Yeast Extract, 3g.; K_2HPO_4, 5 g.; tributyrin emulsion, 50 g.; agar, 15 g.; distilled water, 1,000 ml., pH 5.5) and on the same basal medium with tributyrin replaced by Nile blue sulfate-saturated butterfat.

Effect of yeasts on micrococci. Two selected yeast cultures (B6 and B7) were grown with *M. caseolyticus* culture (Mc32) in milk at pH 4.9 and 6.4 to show the influence of the yeasts on the growth of the micrococci, especially under acid conditions. Also, autolysates of washed yeast cells were tested for their effect on the growth of three species of *Micrococcus.*

Tests on odor production. Seven kinds of culture media were inoculated with pure cultures of yeasts and micrococci from brick cheese and observed for the sweaty odor characteristic of the brick cheese smear. These media included: skimmilk, whole milk, Bacto-Casitone (0.5%) broth plus Bacto-Yeast Extract (0.01%), Bacto-nutrient broth with and without 3% melted and filtered butterfat, and a broth containing 0.5% Bacto-Tryptone and 0.3% beef extract, with

and without 3% melted and filtered butterfat. Also, whole milk was inoculated with a mixture of two yeasts and a micrococcus. The lower volatile saturated fatty acids produced by some of the cultures were estimated by partition chromotography by the methods of Ramsey and Patterson *(13)* and Claborn and Patterson *(5)*.

RESULTS

The 136 cultures of film yeasts isolated from cheese smears and brines fall into four groups on the basis of the shape of the cells and the type of film formed on liquid media. No attempt was made to identify the yeasts, but a representative (B7) of the most commonly occurring group and a yeast that was both proteolytic and lipolytic (B6) were selected for further study. The majority of the film yeasts, as represented by B7, were neither proteolytic nor lipolytic. Iya and Frazier *(9)* had identified the predominant film yeasts of smears as species of *Mycoderma*.

The 329 cultures of micrococci isolated from cheese smears were classified into several groups, representatives of which were studied in detail to identify them as to species. The species found most often was a colorless variant of *M. varians* (Mv), which made up 80% of all cultures isolated and was found in all cheese smears tested. Found less frequently were *M. caseolyticus* (Mc) and *M. freudenreichii* (Mf).

Associative growth of film yeasts and micrococci. Since the film yeasts of cheese smears have been found by Iya and Frazier *(9)* to have a stimulating effect on *B. linens*, it was suspected that the micrococci of the smear might be stimulated in a similar manner. All of the micrococci isolated from the smears were found able to grow at pH levels of 4.8 to 5.5 in broth, a pH range similar to that on the surface of freshly made brick cheese, but developed at an increased rate as the pH approached neutrality. Therefore, the reduction of the acidity of the cheese at the surface, accomplished by film yeasts according to Iya and Frazier, might be expected to favor the growth of the micrococci. This was demonstrated by growing a culture of *M. caseolyticus* (Mc32) in skimmilk at 30° C. with and without the film yeasts B6 and B7. The milk was at pH 6.4 or was adjusted to pH 4.9 to simulate the pH of the surface of a freshly dipped cheese. Counts of both micrococci and film yeasts were made with tryptone (0.5%), glucose (0.1%), beef extract (0.3%) agar and of film yeasts alone with agar made by adding 1.5% agar to the special Iya broth described in *Methods*.

The results in Table 1 show that the micrococcus by itself or in combination with film yeasts grew much better in skimmilk at pH 6.4 than at pH 4.9. The micrococcus alone made only a 1.7 fold increase in 56 hours in milk at pH 4.9, but a 6.9 fold increase in the presence of film yeasts. That the stimulating effect of the yeasts was not entirely due to destruction of lactic acid was indicated by the better growth of the micrococcus in milk at pH 6.4 in the presence of film yeasts than in their absence. The film yeasts seemed to obtain no benefit from growing with the micrococcus.

TABLE 1

Growth of micrococcus Mc 32 and film yeasts B6 and B7 separately and together

Flask[b]	Plating medium[a]	Initial pH of milk	Number of organisms per ml. (× 1,000) after incubation at 30° C. for 0 hours (a) Micrococci	0 hours (b) Yeasts	33 hours (c) Micrococci	33 hours (d) Yeasts	56 hours (e) Micrococci	56 hours (f) Yeasts	Ratio e:a	Ratio f:b
A	TGE	6.4	5,400		27,000		87,000		16.1	
B	TGE	4.9	3,200		3,200		5,400		1.7	
C	Iya	6.4		110		500		1,700		15.5
D	Iya	4.9		120		340		930		7.8
E	TGE	6.4	1,850	50	18,770	230	70,560	440	38.1	8.8
E	Iya	6.4		50		230		440		8.8
F	TGE	4.9	1,500	100	3,330	170	10,400	600	6.9	6.0
F	Iya	4.9		100		170		600		6.0
G	TGE	6.4	0	0	0	0	0	0	–	–

[a] TGE = tryptone glucose beef-extract, which grows both micrococci and film yeast.
Iya = Iya's agar acidified to pH 4.1 with lactic acid, which grows only film yeasts.
[b] A and B inoculated with micrococcus Mc 32.
C and D inoculated with film yeasts, both B6 and B7.
E and F inoculated with micrococcus Mc 32 and film yeasts B6 and B7.
G uninoculated.

To demonstrate that the stimulating effect of the film yeasts on the growth of the micrococci was due to more than the reduction of the acidity by the film, four cultures of micrococci, representing the three species of *Micrococcus* found on cheese smears, were grown in skimmilk at room temperature with and without added autolysate of each of two film yeasts. The results in Table 2 indicate that the autolysates of both film yeasts were stimulatory to the growth of all four cultures of micrococci tested. The stimulation is attributed to available nitrogen compounds and accessory food substances in the autolysates of the yeasts.

Odors of cultures of smear organisms. Brick cheese that has developed a smear normally has a characteristic odor that has been described as sweaty, a term used also to describe the odor of some of the higher volatile fatty acids, especially isovaleric acid. An effort was made to determine whether the micrococci or film yeasts or combinations of them could be demonstrated to be responsible for the brick cheese odor. Pure cultures of five of the micrococci and the two

TABLE 2

Effect of two film yeast autolysates on the growth of micrococci in skimmilk at room temperature

Micrococcus	Numbers of micrococci per ml. (× 1,000) Control[a] 0 hr.	Control[a] 48 hr.	B6 autolysate[b] 0 hr.	B6 autolysate[b] 48 hr.	B7 autolysate[c] 0 hr.	B7 autolysate[c] 48 hr.
Mc 11	280	640,000	190	1,400,000	330	1,300,000
Mc 32	6,900	1,100,000	6,700	2,200,000	9,600	1,700,000
Mf 43	10,000	3,800,000	17,000	7,800,000	14,000	6,400,000
Mv 22	220	490,000	220	1,200,000	190	800,000

[a] Control = 5 ml. sterile water added to 50 ml. skimmilk.
[b] B6 autolysate = 5 ml. B6 autolysate added to 50 ml. skimmilk.
[c] B7 autolysate = 5 ml. B7 autolysate added to 50 ml. skimmilk.

film yeasts were grown for 2 weeks at room temperature in various media to test for the development of the sweaty odor characteristic of the cheese smear. The most commonly found micrococcus of the smear, *M. varians,* did not produce any sign of the odor when grown in skimmilk, whole milk, or Bacto-Casitone plus yeast extract. Cultures of *M. caseolyticus* and *M. freudenreichii* produced a sweaty odor in one or more of these media. The proteolytic and lipolytic film yeast (B6) produced a sweaty odor in skim and whole milk, whereas the nonproteolytic and nonlipolytic film yeast (B7) did not. *M. caseolyticus* did not cause the sweaty odor when grown in peptone or tryptone nutrient broth, with or without added butterfat. A combination of the two film yeasts and *M. caseolyticus* (Mc32) yielded a marked sweaty odor when grown in whole milk, an odor stronger than that produced by the micrococcus or film yeast alone. It was observed that the sweaty odor could be produced in either skimmilk or whole milk but usually more strongly in the latter.

An analysis by partition chromatography for volatile acids produced by the micrococci and yeasts in pure culture in Bacto-Casitone containing 0.01% Bacto-Yeast Extract showed that the yeast and micrococcus cultures differed in their ability to form butyric, propionic, and acetic acids in the medium. The strains of *M. caseolyticus* yielded considerably more butyric acid than the other organisms tested. Levels of acetic and propionic acid were comparatively low except in the instance of the proteolytic and lipolytic film yeast (B6). Higher volatile acids were not present in measurable amounts.

Because cultures of micrococci streaked on milk agar plates often gave off an odor resembling that of brick cheese, an attempt was made to identify some of their odoriferous compounds. About 20 plates of each culture were flooded with 1% hydrochloric acid, and the washings were pooled for extraction with ether and fractionation by chromatography. Three fractions were collected, two of which were identified, one as butyric and the other as acetic acid. The third fraction, which was composed of higher fatty acids than butyric, gave a residue, on evaporation of the solvent, the odor of which resembled that of the streak plate cultures. The odor was pungent and resembled that of butyl alcohol plus the odor of the fatty acids, valeric and caproic. It is evident, then, that the micrococci can produce odors like those of the smear on ripening brick cheese.

Flavor of surface-inoculated cheeses. The real test of the ability of the pure cultures isolated from brick cheese smears to affect the flavor of the cured cheese was a series of experiments in which the surfaces of cheeses were inoculated with one or more cultures by the procedures described under *Methods.* Preliminary experiments had shown that autoclaved cheese vat brine or 22% sodium chloride brine was not as satisfactory as pasteurized 22% brine for salting the cheese. Inoculation of the surface of cheeses with a pure culture of either film yeast (B6 or B7) did not bring the flavor of these cheeses up to that of the control cheeses, even though the yeasts grew well and some micrococci appeared after 7 to 9 days on the cheeses inoculated with film yeasts. The addition of the two film yeasts together seemed to cause some improvement in flavor, which, however, was not as good as that of the control cheeses.

Cheeses inoculated with *B. linens* alone and with *B. linens* and the two film yeasts did not develop the characteristic brick cheese flavor possessed by the control, although the flavor was mild and pleasant. Contrary to expectations, no Limburger flavor developed upon the addition of *B. linens*, but this organism did not grow on the surface according to microscopically obtained evidence.

Cheeses inoculated with each of two strains of *M. caseolyticus* but no film yeasts did not show any typical brick cheese flavor and usually were inferior to the control cheeses in flavor. Microscopic tests on the smear showed that the micrococci decreased in numbers throughout the curing period.

The foregoing experiments had demonstrated that film yeasts should precede the micrococci in order to get appreciable growth and activity of the latter. Therefore, a series of cheeses was inoculated with both film yeasts and with single cultures of representatives of the three species of *Micrococcus* previously identified. The results in Table 3 indicate that a brick cheese flavor was obtained more often with strains of *M. caseolyticus*, but that *M. freudenreichii* and *M. varians* could contribute this flavor.

TABLE 3

Effect upon the flavor of brick cheese of adding various micrococci plus a pair of film yeasts to the surface

Micrococcus added[a]	Flavor grade[b]	Total grade	Brick cheese flavor	Description of flavor
Mc 11	4.0	3.7	−	salty, acid, fermented
Mc 32	4.0	3.7	−	salty, sl. bitter, sl. acid, fermented
Control	4.0	3.7	−	salty, acid, fermented
Mc 11	3.0	3.3	−	salty, bitter, acid, yeasty, pleasant
Control	2.3	2.0	−	yeasty, pleasant
Mc 11	3.8	4.0	+	salty, sl. acid, yeasty, sl. fermented
Mc 32	3.2	3.3	+	salty, sl. acid, fermented, glutamic
Mc 58	3.8	3.8	++	salty, bitter, acid, glutamic
Mf 15	3.2	3.5	−	salty, sl. acid, yeasty, sl. fermented
Mf 43	3.2	3.3	+++	salty, sl. acid, sl. unclean, yeasty
Mv 22	3.8	3.8	+	salty, bitter, sl. acid, yeasty, fermented
Control	3.2	3.5	−	salty, acid, sl. unclean, fermented
Mc 11	2.7	2.8	++	salty, sl. acid, sl. yeasty, pleasant
Mc 32	2.6	2.6	+	salty, sl. bitter, sl. yeasty, acetic
Mc 58	2.8	3.8	−	salty, sl. acid, sl. yeasty, sl. unclean
Mf 15	3.0	3.1	−	salty, sl. acid, yeasty, acetic
Mf 43	2.9	2.9	−	salty, sl. acid, yeasty, sl. unclean, fruity
Mv 22	2.7	2.7	−	salty, sl. acid, yeasty, acetic
Control	3.6	3.7	−	salty, acid, yeasty, Limburger, acetic

[a] Cheese previously inoculated with a saline suspension containing both B6 and B7 film yeasts.
[b] See *Methods* for numerical grading.

The presence or absence of characteristic brick flavor in the cheese is of primary interest in the data shown. Flavor grade, total grade, and descriptions of flavor are included to indicate that other flavors might mask the brick flavor and that flavor and total grade did not necessarily parallel the intensity of the brick flavor. Microscopic examinations of the smears showed that *M. caseolyticus* and *M. freudenreichii* grew well in the smear during ripening but that *M. varians* did not increase in numbers until about the ninth day.

TABLE 4

Effect upon flavor of brick cheese of adding mixture of six micrococci and a pair of film yeasts together to the surface

	Flavor grade[a]	Total grade	Brick cheese flavor	Description of flavor
Test	4.0	4.0	+	salty, acid, fermented, sl. Limburger
Control	3.2	3.5	−	salty, acid, sl. unclean, yeasty
Test	2.8	3.8	+	salty, acid, yeasty, sl. fermented, sl. Cheddar
Control	3.6	3.7	−	salty, acid, yeasty, Limburger, acetic

[a] See *Methods* for numerical grading.

Since strains of all three species of *Micrococcus* could contribute characteristic flavor to brick cheese by growing in the surface smear, a combination of strains should be effective. A combination of six strains, representing the three species of *Micrococcus*, and the two film yeasts was inoculated onto the surfaces of two lots of cheese, which along with controls were cured as usual. The results in Table 4 show that the combinations of cultures produced brick cheese flavor in both test cheeses, whereas the control cheeses of this lot were devoid of the typical flavor.

DISCUSSION

It is evident from the results that micrococci are the chief organisms concerned in the production of the characteristic odor of the smear of brick cheese and of the flavor given the cheese by that smear and that previous growth of film yeast in the smear intensifies the flavor production. However, the most commonly encountered type of film yeast cannot cause the flavor by itself. The lipolytic and proteolytic yeast used in some of the experiments can contribute flavor, but this type of film yeast apparently is so rare in occurrence (found once in 136 isolations) as to be of little significance. The function of the film yeasts is primarily to improve growing conditions for the micrococci which follow.

The most numerous micrococci in smears, *M. varians*, do not seem to be as active in flavor production as the two other species ordinarily found growing along with it, *M. caseolyticus* and *M. freudenreichii*, but a combination of these micrococci is effective.

The flavoring substances formed by the micrococci of the smear have been shown to include butyric and acetic acids and sometimes propionic acid, but it is the higher volatile fatty acids that are responsible for the sweaty odor of the brick cheese smear, as in the surface taint of butter.

Hastening of development of the smear on cheese results when cultures of smear organisms are rubbed onto the ripening cheese. The selection of film yeasts and micrococci known to produce desirable flavors should help guarantee the prompt production of good flavor in brick cheese.

SUMMARY

Pure cultures of film yeasts and of micrococci were isolated from surface smears of brick cheese and from cheese brines. A culture of the most commonly found film yeast and one that was both lipolytic and proteolytic were selected

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for experiments on associative growth and inoculation of cheese surfaces. The micrococci were found to be predominantly colorless variants of *M. varians*. Next in order of occurrence were *M. caseolyticus* and *M. freudenreichii*. The associative growth of film yeasts and micrococci was studied and attempts were made to ascertain the source and the cause of the typical odor of a brick cheese smear. Experimental cheeses were inoculated on the surface with film yeasts and micrococci, alone or in combinations, to find their effect on flavor.

Growth of micrococci with film yeasts indicated that the yeasts stimulate the growth of the cocci on the surface of a brick cheese by reducing the acidity of the cheese surface and furnishing accessory food substances to the cocci.

M. caseolyticus and *M. freudenreichii*, but not *M. varians*, were found to be able to produce the characteristic sweaty odor of a brick cheese smear when grown in milk and other media, as could the proteolytic and lipolytic film yeast, but not the film yeast most commonly found in smears.

Butyric and acetic acids were identified as products of the growth of cultures of micrococci on milk agar, but the fraction with an odor characteristic of brick cheese smear was one containing higher fatty acids.

Inoculation of cheese surfaces with single cultures or combinations of them gave the following results: For the most part the addition of the two film yeasts to the surface, alone or combined, or the separate addition of *B. linens* or of single cultures of micrococci did not improve the flavor of the brick cheese. The addition of *B. linens* plus the two film yeasts neither improved the flavor nor produced a Limburger flavor. Brick cheese flavor was detected in seven of 15 cheeses inoculated with separate strains of micrococci combined with the two film yeasts. The typical flavor was found most frequently in cheeses to which *M. caseolyticus* had been added, but one cheese inoculated with *M. freudenreichii* had a very pronounced brick cheese flavor. In both trials in which a combination of six micrococci, which included the three species, and the two film yeasts were added to the cheese, brick cheese flavor was produced.

It is concluded that micrococci, especially combinations of them, in the smear are important in the production of the characteristic brick cheese flavor, and that this flavor is accentuated by the previous growth of film yeasts.

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